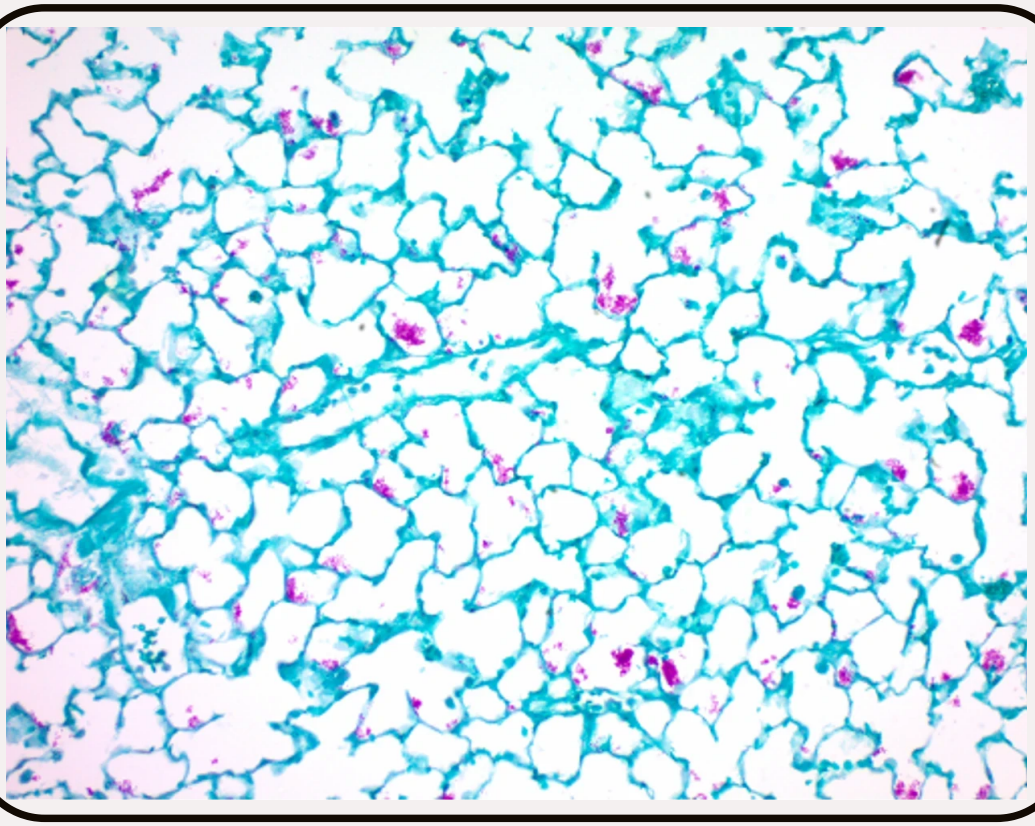
प्रयोगSHALA

ISSUE 1

2081 SHRAWN



prithak

Issue 1 Shrawn 2081

प्रयोगSHALA

A monthly compendium

CRISPR-CAS9 GENOME EDITING: FUTURE OF MEDICINE

Prithakraj Kandel
Bsc.MLT, 1st year
Maharajgunj Medical Campus

Introduction

CRISPR-Cas9 is the most promising technique in biotechnology and molecular biology of the past decade. CRISPR-Cas9 system represents genome editing technology that has revolutionized molecular biology, due to its precise and site-specific gene editing capabilities, which allow for an unprecedented level of control in manipulating the genetic information of an organism. The RNA-guided nucleases from CRISPR-Cas systems are currently regarded as the most reliable tools for genome editing and engineering. The new level of targeting, efficiency and ease of use of genomic editing tools created by the CRISPR-Cas9 system have overthrown gene editing technologies like TALENs (Transcription Activator-Like Effector Nucleases) and ZFNs (Zinc Finger Nucleases).

History

CRISPRs were first identified in *E. coli* in 1987 by a Japanese scientist, Yoshizumi Ishino, and his team .While studying isozyme conversion of alkaline phosphatase unexpectedly discovered a pattern of repeated sequences in the bacterial genome. However, due to the lack of sufficient DNA sequence data, the function of these arrays remained a mystery .Basically these refer to short, repeated sequences of DNA nucleotides found within the genome of prokaryotes. These sequences are the same when read from 5' to 3' on one strand of DNA from 5' to 3' on the complementary strand, and are therefore described as palindromic repeats.

The first experimental information about the mechanism of action of the CRISPR system was obtained in 2007 in the studies of two French food scientists, Rodolphe Barrangou and Philippe Horvath, who worked with yoghurt cultures of bacteria *Streptococcus thermophilus* for the Danish company Danisco . Due to the company's rich collection of bacterial strains collected since the 1980s, scientists have been able to trace the historical course of the bacterial acquisition of spacers at the CRISPR locus in response to viral attacks by bacteriophages. The addition of new spacers in this work caused acquired immunity to the corresponding new types of bacteriophages in *S. thermophilus*: observation which subsequently led to the authors concluding that CRISPR system is associated with adaptive immunity of bacteria.

In 2012, George church Emmanuelle Charpentier, in collaboration with a prominent structural biologist, Jennifer Doudna of the University of California (UC), Berkeley, had transformed the CRISPR immune system into a tool that could edit genomes with great ease .They received the 2020 Nobel Prize in Chemistry for discovering *"One of gene technology's sharpest tools: the CRISPR/Cas9 genetic scissors"* .

Mode of action.

During a viral infection, unique sequences that are nestled in between the the palindromic repeats: referred to as spacers are bits of foreign dna that originate from mobile genetic elements, or MGEs, such as bacteriophages, transposons, or plasmids that have previously infected the prokaryote. Bacteria acquire a small piece of the foreign viral DNA,and integrate it into the CRISPR locus to generate CRISPR arrays. CRISPR arrays consist of duplicate sequences, which are the palindromic repeats belonging to the bacterial genome, flanked by variable sequences, or spacers, which again are from the foreign genetic elements. In this way, bacteria retain a memory of past infection. To fend off an invader, short RNA sequences transcribed from the CRISPR spacer regions (CRISPR RNA or crRNA) are processed from larger pre-crRNA transcripts. The crRNA then forms a complex with a trans-activating CRISPR RNA (tracrRNA) and Cas enzyme .This complex, led to its target by the crRNA

homology where it binds and cuts the invading DNA. Cas9 requires a specific protospacer adjacent motif (PAM) to cut the DNA. The protospacer is the part of the crRNA sequence that is complementary to the target sequences incorporated into the bacterial genome. The PAM varies depending on the bacterial species of the Cas gene. The Cas9 nuclease from Streptococcus pyogenes recognizes a PAM sequence of NGG directly 3 ′ of the target sequence in the target DNA, on the complementary strand. The PAM may facilitate the formation of the RNA:DNA hybrid between the crRNA and the target DNA. In doing so, it will neutralize the virus, because its genome can no longer be transcribed properly to create more viral particles, so infection is not impossible.

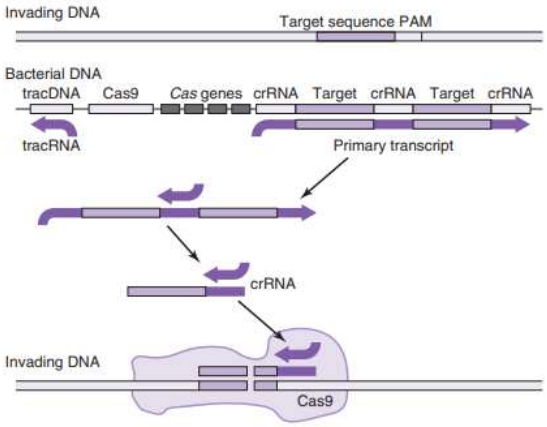


Fig : Organization of CRISPR-CAS9 system in bacteria (source : Buckingham Lela; Molecular Diagnostics Fundamentals, Methods, and clinicaI Applications,3e)

Genome editing using CRISPR-CAS9 system

In bacteria, the crRNA and tracrRNA are separate molecular entities. The first major breakthrough arrived when it was realized that the roles of these molecules could be combined into a single molecule by fusing them together with a linker to generate something called single guide RNA, or sgRNA, which can be synthesized in the lab. If the sgRNA complexes with a Cas9 protein, this two-component system will be able to cleave DNA just as the three-component system does in bacteria. It was then possible to determine any sequence of about 20 base pairs as a target for editing, and all that to be done is to synthesize the appropriate sgRNA with the complementary sequence, and insert that into a cell along with the Cas9 protein which has been sourced from Streptococcus pyogenes. The complex will form, read the DNA until it finds the appropriate sequence along with a PAM sequence, binding will occur, and DNA will be cleaved at precisely the desired location. Cas9 has two domains, and each one will snip one of the DNA strands. After the incision is made, the natural DNA repair mechanism is enacted for the target DNA. The cleaved dsDNA can undergo repair via two routes. After the incision is made, the natural DNA repair mechanism is enacted for the target DNA. The cleaved dsDNA can undergo repair via two routes. Either by homology-directed repair, abbreviated as HDR, or by non-homologous end joining, abbreviated as NHEJ. The NHEJ pathway repairs double-strand breaks in DNA by directly ligating without the need for a homologous template, which means a DNA strand with similar sequence that can act as a template. The NHEJ mechanism can also introduce insertion or deletion of specific sequences at the joining ends, thus creating indels. Indels are DNA strands with either an insertion or deletion of nucleotide sequences. Thus, NHEJ produces DNA strands with non-uniformity in size. The other route of repair, the HDR pathway, is commonly found in bacterial and archaeal cells, while the NHEJ pathway is more common in a eukaryotic domain. The HDR process, although more complex than NHEJ, uses a homologous DNA template. The homologous DNA template has homology to the adjacent sequences surrounding the site of cleavage to incorporate new DNA fragments. The template guides the repair process, and lowers the possibility of errors. Since there is no insertion or deletion of nucleotide sequences, the HDR pathway maintains uniformity in the size of the resulting dsDNA, unlike NHEJ.

Potential applications

The discovery of CRISPR-Cas9 has ushered in a new era of precision genome editing, offering unprecedented possibilities for tackling complex diseases .A groundbreaking study conducted by researchers at the University of Pittsburgh and the Lewis Katz School of Medicine at Temple University has harnessed the power of CRISPR/Cas9 to combat HIV infection. Although conventional anti-retroviral therapies capable of suppressing viral replication. But once the virus gets converted to its proviral form, conventional therapies are ineffective in targeting the virus. The provirus resides within the immune cells and continues to make copies of itself using the immune cell machinery, and the immune cells fail to target the proviral latent reservoir which presents the risk of viral rebound or relapse of the disease .By precisely targeting and eliminating HIV-infected human immune cells in mice, scientists have demonstrated the technology's potential to eradicate the virus. This pioneering research marks a significant step forward in the quest for an HIV cure and paves the way for future human trials.

Schwank et al used CRISPR/Cas9 to investigate the treatment of CF. Using adult intestinal stem cells obtained from two patients with CF, they successfully corrected the most common mutation causing CF in intestinal organs. They demonstrated that once the mutation had been corrected, the function of the CF transmembrane conductor receptor (CFTR) was restored.

T-cell genome engineering using CRISPR-CAS9 technology can be used in treating cancers, primary immune deficiencies and autoimmune diseases. Recently a more effective approach in human $CD4^+$ T-cells manipulation based on the CRISPR/Cas9 system has been reported. Their technique allowed experimental and therapeutic editing of the genome in primary human T-cells. They demonstrated T-cells could be manipulated to prevent expression of the carcinogenic protein PD-1.

It has also been employed to resolve hereditary hematological malignancies. Frangoul *et al* experimented on CD34+ cells of two persons ,one with SCD and one with Transfusion-dependent β-thalassemia (TDT) edited with CRISPR-Cas9 targeting the *BCL11A* enhancer . *BCL11A* is a transcription factor that represses *γ*-globin expression and fetal hemoglobin in erythroid cells.. More than a year later, both patients had high levels of allelic editing in bone marrow and blood, increases in fetal hemoglobin that were distributed pancellularly, transfusion independence, and elimination of vaso-occlusive episodes (in the patient with SCD).

Conclusion

The discovery of CRISPR–Cas9 has led to a revolution in the field of genomic manipulations. New opportunities have opened up in multiple areas of biomedicine, such as molecular diagnostics of infectious and non-infectious diseases such as genotyping of bacterial strains, detection of viruses, treatment of hereditary hematological malignancies and identification of genetic mutations in circulating extracellular DNA in patients with lung cancer, as well as in the development of a potentially new method of immunization, DNA vaccines .The proper development of legislation and ethical principles in the application of the CRISPR–Cas9 system in genome editing is necessary to utilize this technology for benefit of mankind .

Limitations

It has already been shown that CRISPR/Cas9 technology can alter the genome of human embryos which could prove useful in the preimplantation treatment of genetic diseases. However, any genetic modification of the germline would be permanent and the long-term consequences are unclear. Many oppose germline modification under any circumstances, reasoning that an eventual consequence could be non-therapeutic genetic enhancement.

References

1.Buckingham Lela; **Molecular Diagnostics Fundamentals, Methods, and clinicaI Applications,3e**

2.Shino Y., Shinagawa H., Makino K., Amemura M., Nakatura A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isoenzyme conversion in *Escherichia coli*, and identification of the gene product. *J. Bacteriol*. 1987;169:5429–5433. doi: 10.1128/jb.169.12.5429-5433.1987

3.Hsu PD, Lander ES, Zhang F**. Development and Applications of CRISPR-Cas9 for Genome Engineering**. *Cell* 2014;157:1262–78. 10.1016/j.cell.2014.05.010

4.Ishino, Y. *et al*. **History of CRISPR-Cas from encounter with a mysterious repeated sequence to genome editing technology**. *Journal of Bacteriology*, 200, 7 (2018).

5.Frangoul H, *et al*. **CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β-Thalassemia**. *N Engl J Med.* 2021 Jan 21.

TRANSFUSION RELATED ACUTE LUNG INJURY AND ITS PATHO-PHYSIOLOGY

Bishal Paudel
Bsc.Mlt, 1st year
Maharajgunj Medical Campus

The term Transfusion-related Acute Lung Injury was coined in 1985 to describe acute respiratory distress syndrome(ARDS) after transfusion. When other ARDS risk factors are absent; TRALI cases are predominantly associated with donor leucocytes antibodies ie. Also called immune mediated TRALI. In 2001, plasma from multiparous donors was implicated in TRALI in a randomized controlled trial in Sweden.

Anti HLA/HNA antibodies are implicated in Immune mediated TRALI. They bind to antigens expressed on pulmonary endothelia to initiate acute inflammation on lungs leading to increased permeability pulmonary edema. Mainly multiparous women develops these antibodies through exposure to fetal blood. The cases of TRALI are seen decreased using male-predominant plasma.

Pathophysiology

A two-hit model of neutrophil-mediated proinflammatory endothelial activation and pulmonary endothelial damage that requires adherence and chemokine release has been described.
Inflammation is commonly an important first hit in the pathogenesis of TRALI. Lung pathology in fatal TRALI cases and in experimental models has revealed the prominent presence of neutrophils in lung capillaries. In transfusion recipients, increased pretransfusion IL-8 was associated with greater risk for TRALI. Inflammation in the lungs was found in transfused cardiac surgery patients. Cytokines, chemokines, and cell-derived particles in the supernatant of red blood cell units may cause a proinflammatory response in the recipient. Transfusion of donor antibodies has been found to be the important risk factor in controlled clinical studies and is a well-established cause of TRALI. Direct endothelial damage by complement-fixing antibodies may lead to a neutrophil response. Activated platelets are also important contributors to lung pathology and can induce the formation of neutrophil extracellular traps (NETs). NETs are composed of decondensed chromatin decorated with granular proteins that function to trap extracellular pathogens and, in one study, have been detected in the lungs and plasma of patients with TRALI. These findings indicate that NETs may contribute to lung endothelial injury in TRALI in humans. In addition, one study found that post-transfusion complement activation increases in human TRALI, and the increase may facilitate NET release and pulmonary endothelial injury. The pathophysiology of TRALI is complex. The contributions of other immune inflammatory mediators and cells have been found in experimental studies. such as bioactive lipids that accumulate in stored platelets and macrophages.

There is no specific laboratory tests for TRALI, current diagnosis is based on criteria that depend on clinical information.

ERYTHROBLASTOSIS FETALIS: A SILENT THREAT TO UNBORN BABIES

Uttam Timalsina
Bsc.Mlt, 1st year
Maharajgunj Medical Campus

Introduction

Hemolytic disease of the fetus and newborn (HDFN), also erythroblastosis fetalis, is caused by the destruction of red blood cells of the neonate or fetus by maternal immunoglobulin G (IgG) antibodies. The formation of maternal antibodies in response to a fetal antigen is called isoimmunization. These antibodies form when fetal erythrocytes that express certain RBC antigens ((usually Rh) that are not expressed in the mother cross the placenta and gain access to maternal blood. This antibody response may destroy fetal red cells leading to hemolysis, the release of bilirubin, and anemia.

Following maternal exposure to Rh D-positive blood, B-lymphocyte clones that recognize the RBC antigen are established. The primary maternal immune response is the production of IgM isotype. The secondary immune response follows repeat exposure to as little as 0.03 mL of Rh-positive cells. Maternal anti-D (IgG) antibodies cross the placenta and attach to Rh antigens on fetal RBCs. RBC destruction occurs by lysis of antibody-coated RBCs by macrophage lysosomal enzymes. The fetus initially responds to the subsequent anemia and tissue hypoxia through reticulocytosis, and a rise in umbilical artery lactate indicates severe fetal anemia. Erythroblastosis fetalis results when RBC destruction exceeds production.

History

In 400 BC, Hippocrates, considered to be the founder of modern medicine, first described a fetus carnosus or "fleshy fetus," which is now believed to have been hydrops fetalis (result due to severe HDFN) .Hippocrates may have described fetal hemolytic disease (erythroblastosis fetalis, hemolytic disease of the newborn), the first recorded report is that of a French midwife in 1609, who reported the birth of twins, the first was grossly edematous (hydrops fetalis) and died promptly, the second in good condition at birth, became deeply jaundiced (icterus gravis) and died a few days later . Dozens of similar reports were made in the centuries there after, reporting on "universal edema of the fetus," "fetal leukemia," "unusual blood forming foci in the liver," and a "familial incidence." . It was not until the efforts of Landsteiner starting with identification of ABO blood group system in 1900 , followed by Rh in the 1940s that the pathophysiology of the disease was uncovered. A year later, Levine and Stetson published on pregnancy-related alloimmunization . Cumulatively, these developments led to a great decrease in the prevalence and severity of the disease, through timely identification and prompt treatment.

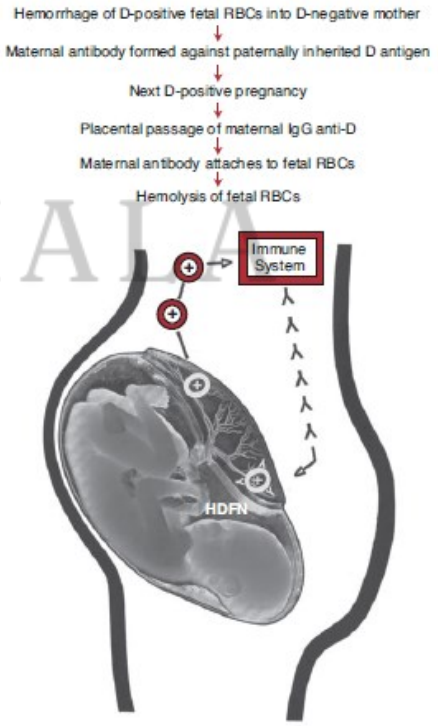

Hemorrhage of D-positive fetal RBCs into D-negative mother
Maternal antibody formed against paternally inherited D antigen
Next D-positive pregnancy
Placental passage of maternal IgG anti-D
Maternal antibody attaches to fetal RBCs
Hemolysis of fetal RBCs
Immune System
HDFN

Figure : Pathogenesis of hemolytic disease of the fetus and newborn caused by maternal alloimmunization and incompatibility between the fetus and mother.

(Source: Denise M. Harmening ,Modern Blood Banking & Transfusion Practices)

Prenatal identification and Diagnosis

1. **Antiglobulin tests**

   The antiglobulin test, or Coombs test, is a laboratory technique used to detect antibodies that are attached to red blood cells. There are two main types:

   Direct Antiglobulin Test (DAT): This test directly checks if there are antibodies stuck to the surface of your red blood cells. It's used to diagnose conditions like autoimmune hemolytic anemia and hemolytic disease of the newborn.

   .

Indirect Antiglobulin Test (IAT): This test looks for antibodies in your blood plasma that could potentially attach to red blood cells. It's often used before blood transfusions to check compatibility and during pregnancy to screen for antibodies that could harm the baby.

2. Amniotic fluid analysis

This involves examining the amniotic fluid surrounding the baby for bilirubin levels. Bilirubin is a breakdown product of red blood cells. High levels of bilirubin in the amniotic fluid suggest hemolysis in the baby, indicating erythroblastosis fetalis.

3. Doppler sonography

While primarily used to assess fetal well-being, Doppler sonography can indirectly provide information about erythroblastosis fetalis by evaluating blood flow patterns in the umbilical cord and fetus. Abnormal blood flow patterns may suggest complications associated with the condition

4. Biological assays: Antibody-dependent cellular-cytotoxicity (ADCC) assay, may be employed to increase the positive predictive value .

Treatment Prevention of Erythroblastosis Fetalis

1. Prevention

-Rh Immune Globulin (RhoGAM): Administered to Rh-negative mothers during pregnancy and after childbirth, this medication prevents the formation of antibodies that can harm the fetus.

2. Treatment for the Unborn Baby

-Intrauterine Blood Transfusion: In severe cases, blood transfusions can be performed directly into the fetus's bloodstream to replace damaged red blood cells.

-Early Delivery: If the condition is severe and the fetus's lungs are mature enough, early delivery may be necessary.

3.Treatment for the Newborn

-Phototherapy: Exposure to special lights can help break down bilirubin, a byproduct of red blood cell breakdown.

-Exchange Transfusion: This involves replacing the baby's blood with donor blood to remove harmful antibodies and provide healthy red blood cells.

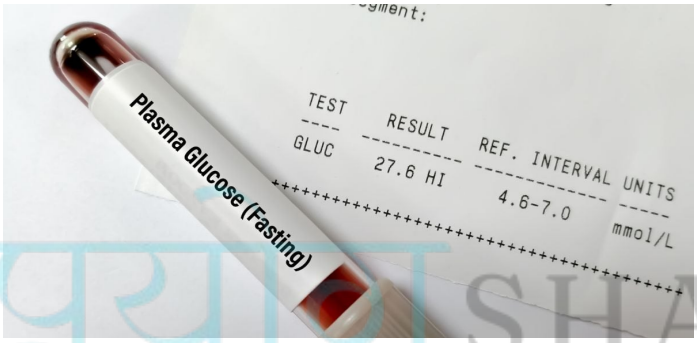
-Supportive Care: Treatment may also include medications to manage breathing difficulties and other complications.

प्रयोगSHALA

CHALLENGING THE NORM: POSTPRANDIAL GLUCOSE LEVELS LOWER THAN FASTING VALUES

Roshan Pokhrel
Bsc.Mlt, 1st year
Maharajgunj Medical Campus

In clinical laboratories, daily numerous blood samples are processed for estimation of fasting blood glucose (FBG) level and postprandial blood glucose (PPBG) level. It is found in some cases that postprandial level of blood glucose is remarkably lower than that of fasting level. Being unsatisfied with the laboratory result, patient or sometimes clinician wants to recheck the blood glucose level in next laboratory. This is because of common perception that PPBG level must be higher than FBG level. But the repeated investigation yields similar type of result. FBG may be higher than the PPBG in both diabetics and healthy population. Many modifiable factors may be the cause for this laboratory finding along with underlying conditions of such patient.



Blood glucose level primarily depends upon individual characters like type and quantity of food intake, physical activity and the body's metabolic response. Pre-analytical factors that affects blood glucose level are smoking, caffeinated drinks, use of hypoglycemic drugs, heavy exercise, anxiety and delay in sample processing. Careful attentions to these modifiable factors by both clinician and laboratory staff are essential to ensure accurate glucose measurement. The organ that is responsible for fasting value of blood glucose is liver whereas it is the pancreas that is responsible for PPBG value. Several hours after dinner, blood glucose level drops leading to decrease in insulin level and rise in glucagon level. Glucagon is responsible for maintaining adequate blood glucose level in fasting condition via activation of metabolic pathways like gluconeogenesis and glycogenolysis in liver. Higher FBG level is due to increase in glucagon to insulin ratio as seen in diabetes, where liver is involved in excess glycogen breakdown and gluconeogenesis. Despite having sufficient insulin, individual may have higher FBG value which is mainly due to insulin resistance that is the commonest cause of impaired fasting glucose tolerance and diabetes mellitus. . Apart from this underlying disturbed homeostatic response of person, various modifiable factors have effect on FBG level. Higher FBG is mainly due to high carbohydrate meal at bedtime or not enough diabetic medication. The anxious individual with disturbed sleep may also have high FBG.

BLOOD BANKING AND AUTO-AGGLUTINATION : MY EXPERIENCE

Ragini Shrestha
Bsc.Mlt, 1st year
Maharajgunj Medical Campus

Blood bank is of immense importance in the health sector. The common people may think it is the storehouse of blood bags and nothing more. But like there is a saying people will just ignore it unless they know its value. While it's just my first year and my first posting in blood bank I got to know that even a single mistake can lead to condition that might lead to people's death. There is a lot more to discuss but the case that got my nerves was autoagglutination. So what actually is autoagglutination and what is its clinical importance? To discuss this I would start from the very basic.

Patients who have blood loss or have required blood transfusion come to blood bank but it's not as easy as it seems. It's not like we go to shop to buy something and instantly the shopkeeper provides us with the list. For this to process first identification is required and we need to confirm the patient's blood group and then we can provide them with blood bags
but it is not always easy what if the patient's blood group is misleading or confusing like that in case of autoagglutination while in the normal case when we do forward blood grouping we check for agglutination and determine blood group but here in autoagglutination the sample is itself agglutinated as I have shown and it becomes challenging for us to determine blood group.

Fig 1. Autoagglutination

The phenomenon of autoagglutination was first noted by Klein in 1890. It consists in the accumulation of erythrocytes with serum or citrated plasma of the same blood. Actually in normal sample when we do forward grouping we add antisera on slide on top of which we add the cell suspension of the sample. Then we check for agglutination but in case of autoagglutination the cell suspension seems to be agglutinated by itself without keeping any antisera
(I carried out this procedure and got the result as shown in fig 2).

Fig .2

Autoagglutination can be caused by various factors. It may be due to auto immune hemolytic anemia. Sometimes this could be the result of receiving mismatched blood bags(like person with AB positive blood group receiving A positive blood group) during blood transfusion. In some cases it may be the cause of underlying diseases like multiple myeloma and macroglobulinemia.

When it is likely impossible to determine blood group easily and the patient needs immediate transfusion then group O packed red cells should be used
(Modern blood banking and transfusion practice).The challenge does not just end in determining the blood group. We need to perform compatibility testing like cross matching, serology testing of donors sample and so on.

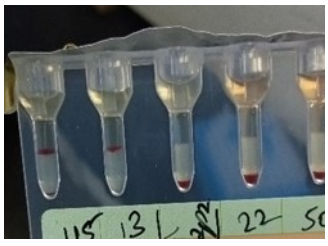


Fig . 3

Fig.3 demonstrates the gel card method of major cross matching in which the first two gel card shows incompatibility while the rest three is compatible.
Here each and every step is important and crucial in saving the peoples lives!